CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as Express Mail in an envelope addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date appearing below.

"Express Mail" mailing label number: EV 341026164 US

August 26, 2003 (Date) By Marcia R. Jansen (Marcia R. Jansen)

APPLICATION FOR LETTERS PATENT

Applicant(s): ffrench-Constant, Richard H.
Bowen, David
Rocheleau, Thomas
Waterfield, Nicholas

Title: DNA SEQUENCES FROM *PHOTORHABDUS LUMINESCENS*

Attorney's Docket No.: 61,645B DIV1

DNA Sequences from *Photorhabdus luminescens*

Related Applications

This application claims priority from United States Provisional Patent Application Ser. No. US 60/191806 filed March 24, 2000.

Background of the Invention

As reported in WO98/08932, protein toxins from the genus Photorhabdus have been shown to have oral toxicity against insects. The toxin complex produced by *Photorhabdus luminescens* (W-14), for example, has been shown to contain ten to fourteen proteins, and it is known that these are produced by expression of genes from four distinct genomic regions: tca, tcb, tcc, and tcd. WO98/08932 discloses nucleotide sequences for many of the native toxin genes.

Of the separate toxins isolated from *Photorhabdus luminescens* (W-14), those designated Toxin A and Toxin B have been the subject of focused investigation for their activity against target insect species of interest, for example corn rootworm. Toxin A is comprised of two different subunits. The native gene tcdA (SEQ ID NO:1) encodes protoxin TcdA (see SEQ ID NO:1). As determined by mass spectrometry, TcdA is processed by one or more proteases to provide Toxin A. More specifically, TcdA is an approximately 282.9 kDA protein (2516 aa) that is processed to provide TcdAii, an approximately 208.2 kDA (1849 aa) protein encoded by nucleotides 265-5811 of SEQ ID NO:1, and TcdAiii, an approximately 63.5 kDA (579 aa) protein encoded by nucleotides 5812-7551 of SEQ ID NO:1.

WO 01/11029 discloses nucleotide sequences that encode TcdA and TcbA and have base compositions that have been altered from that of the native genes to make them more similar to plant genes. Also disclosed are transgenic plants that express Toxin A and Toxin B.

We have observed that heterologous expression of Toxin A does not afford the level of oral toxicity to insects that is observed for the native toxin. It would be very valuable if means could be found to enhance the level of toxicity of heterologously expressed Toxin A.

Summary of the Invention

The present invention provides nucleotide sequences for two genes, *tcdB* and *tccC2*, from the tcd genomic region of *Photorhabdus luminescens* W-14. These sequences were not previously known. We have discovered that coexpression of *tcdB* and *tccC2* with *tcdA* in heterologous hosts results in enhanced levels of oral insect toxicity compared to that obtained when *tcdA* is expressed alone in such heterologous hosts. Coexpression of *tcdB* and *tccC2* with *tcdA or tcbA*, or with any other functionally equivalent toxin gene in the same family as *tcdA* and *tcbA*, to provide enhanced oral insect activity falls within the scope of the invention.

Summary of the Sequences

SEQ ID NO: 1 is the DNA sequence for *tcdA* from Photorhabdus luminescens W-14.

SEQ ID NO: 2 is the amino acid sequence for TcdA from Photorhabdus luminescens W-14.

SEQ ID NO: 3 is the DNA sequence for *tcdB* from Photorhabdus luminescens W-14.

SEQ ID NO: 4 is the amino acid sequence for TcdB from Photorhabdus luminescens W-14.

SEQ ID NO: 5 is the DNA sequence for *tccC2* from Photorhabdus luminescens W-14.

SEQ ID NO: 6 is the amino acid sequence for TccC2 from from Photorhabdus luminescens W-14.

SEQ ID NO: 7 is the DNA sequence for *tcbA* from from *Photorhabdus luminescens* W-14.

SEQ ID NO: 8 is the amino acid sequence for TcbA from Photorhabdus luminescens W-14.

Detailed Description of the Invention

It is preferred for the nucleic acids according to the invention to comprise at least one sequence chosen from

(a) the sequences according to SEQ ID NOS: 3 and 5.

(b) at least 14 base pairs-long partial sequences of the sequences defined under (a),

(c) sequences that hybridize with the sequences defined under (a),

(d) sequences that are at least 70 %, preferably 80 % and even more preferred, 90 % identical to the sequences defined under (a),

(e) sequences that are at least 70 %, preferably 80 % and even more preferred, 90 % similar to the sequences defined under (a),

(f) sequences that complement the sequences defined under (a), and

(g) sequences that due to the degeneracy of the genetic code, code for the same amino acid sequence as(i.e. are "isocoding" with) the sequences defined under (a) through (e).

The expression "hybridize" as used herein refers to hybridization under the following specified conditions: 5 x SSC; blocking reagent (Roche Diagnostics Inc., Mannheim, Germany), 1 %; N-lauroyl-sarcosine, 0.1 %; SDS (sodium-dodecyl sulfate) 0.02 %; hybridization temperature: 60 °C; first wash step: 2 x SSC at 60 °C; second wash step: 2 x SSC at 60 °C; preferred second wash step: 0.5 x SSC at 60 °C; especially preferred second wash step: 0.2 x SSC at 60 °C.

"Identity" and "similarity" are scored by the GAP algorithm using the Blosum 62 protein scoring matrix (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI).

Expression of the Nucleotide Sequences in Heterologous Microbial Hosts

As biological insect control agents, the insecticidal toxins are produced by expression of the nucleotide sequences in heterologous host cells capable of expressing the nucleotide sequences. In a first embodiment, additional copies of one or more of the nucleotide sequences are added to Xenorhabdus nematophilus, Xenorhabdus poinarii, or Photorhabdus luminescens cells either by insertion into the chromosome or by introduction of extrachromosomally replicating molecules containing the nucleotide sequences.

In another embodiment, at least one of the nucleotide sequences of the invention is inserted into an appropriate expression cassette, comprising a promoter and termination signals. Expression of the nucleotide sequence is constitutive, or an inducible promoter responding to various types of stimuli to initiate transcription is used. In a preferred embodiment, the cell in which the toxin is expressed is a microorganism, such as a virus, a bacteria, or a fungus. In a preferred embodiment, a virus, such as a baculovirus, contains a nucleotide sequence of the invention in its genome and expresses large amounts of the corresponding insecticidal toxin after infection of appropriate eukaryotic cells that are suitable for virus replication and expression of the nucleotide sequence. The insecticidal toxin thus produced is used as an insecticidal agent. Alternatively, baculoviruses engineered to include the nucleotide sequence are used to infect insects in-vivo and kill them either by expression of the insecticidal

toxin or by a combination of viral infection and expression of the insecticidal toxin.

Bacterial cells are also hosts for the expression of the nucleotide sequences of the invention. In a preferred embodiment, non-pathogenic symbiotic bacteria, which are able to live and replicate within plant tissues, so-called endophytes, or non-pathogenic symbiotic bacteria, which are capable of colonizing the phyllosphere or the rhizosphere, so-called epiphytes, are used. Such bacteria include bacteria of the genera Agrobacterium, Alcaligenes, Azospiriilum, Azotobacter, Bacillus, Ciavibacter, Enterobacter, Erwinia, Flavobacter, Klebsielia, Pseudomonas, Rhizobium, Serratia, Streptomyces and Xanthomonas. Symbiotic fungi, such as Trichoderma and Gliocladium are also possible hosts for expression of the inventive nucleotide sequences for the same purpose.

Techniques for these genetic manipulations are specific for the different available hosts and are known in the art. For example, the expression vectors pKK223-3 and pKK223-2 can be used to express heterologous genes in E. coli, either in transcriptional or translational fusion, behind the tac or trc promoter. For the expression of operons encoding multiple ORFS, the simplest procedure is to insert the operon into a vector such as pKK2233 in transcriptional fusion, allowing the cognate ribosome binding site of the heterologous genes to be used. Techniques for overexpression in gram-positive species such as Bacillus are also known in the art and can be used in the context of this invention (Quax et al. In.: Industrial Microorganisms: Basic and Applied Molecular Genetics, Eds. Baltz et al., American Society for Microbiology, Washington (1993)). Alternate systems for overexpression rely for example, on yeast vectors and include the use of Pichia, Saccharomyces and Kluyveromyces (Sreekrishna, In: industrial microorganisms: basic and applied molecular genetics, Baltz, Hegeman, and Skatrud eds., American Society for

Microbiology, Washington (1993); Dequin & Barre, Biotechnology 12:173-177 (1994); van den Berg et al., Biotechnology 8:135-139 (1990)).

Expression of the Nucleotide Sequences in Plant Tissue

In a particularly preferred embodiment, at least one of the insecticidal toxins of the invention is expressed in a higher organism, e.g., a plant. In this case, transgenic plants expressing effective amounts of the toxins protect themselves from insect pests. When the insect starts feeding on such a transgenic plant, it also ingests the expressed toxins. This will deter the insect from further biting into the plant tissue or may even harm or kill the insect. A nucleotide sequence of the present invention is inserted into an expression cassette, which is then preferably stably integrated in the genome of said plant, In another preferred embodiment, the nucleotide sequence is included in a non-pathogenic self-replicating virus. Plants transformed in accordance with the present invention may be monocots or dicots and include, but are not limited to, maize, wheat, barley, rye, sweet potato, bean, pea, chicory, lettuce, cabbage, cauliflower, broccoli, turnip, radish, spinach, asparagus, onion, garlic, pepper, celery, squash, pumpkin, hemp, zucchini, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape, raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tomato, sorghum, sugarcane, sugarbeet, sunflower, rapeseed, clover, tobacco, carrot, cotton, alfalfa, rice, potato, eggplant, cucumber, Arabidopsis, and woody plants such as coniferous and deciduous trees.

Once a desired nucleotide sequence has been transformed into a particular plant species, it may be propagated in that species or moved into other varieties of the same species, particularly including commercial varieties, using traditional breeding techniques.

A nucleotide sequence of this invention is preferably expressed in transgenic plants, thus causing the biosynthesis of the corresponding toxin in the transgenic plants. In this way, transgenic plants with enhanced resistance to insects are generated. For their expression in transgenic plants, the nucleotide sequences of the invention may require modification and optimization. Although in many cases genes from microbial organisms can be expressed in plants at high levels without modification, low expression in transgenic plants may result from microbial nucleotide sequences having codons that are not preferred in plants. It is known in the art that all organisms have specific preferences for codon usage, and the codons of the nucleotide sequences described in this invention can be changed to conform with plant preferences, while maintaining the amino acids encoded thereby. Furthermore, high expression in plants is best achieved from coding sequences that have at least about 35% GC content, preferably more than about 45%, more preferably more than about 50%, and most preferably more than about 60%. Microbial nucleotide sequences which have low GC contents may express poorly in plants due to the existence of ATTTA motifs which may destabilize messages, and AATAAA motifs which may cause inappropriate polyadenylation. Although preferred gene sequences may be adequately expressed in both monocotyledonous and dicotyledonous plant species, sequences can be modified to account for the specific codon preferences and GC content preferences of monocotyledons or dicotyledons as these preferences have been shown to differ (Murray et al. Nucl. Acids Res. 17: 477-498 (1989)). In addition, the nucleotide sequences are screened for the existence of illegitimate splice sites that may cause message truncation. All changes required to be made within the nucleotide sequences such as those described above are made using well known techniques of site directed mutagenesis, PCR, and synthetic gene construction.

For efficient initiation of translation, sequences adjacent to the initiating methionine may require modification. For example, they can be modified by the inclusion of sequences known to be effective in plants. Joshi has suggested an appropriate consensus for plants (NAR 15: 6643-6653 (1987)) and Clontech suggests a further consensus translation initiator (1993/1994 catalog, page 210). These consensuses are suitable for use with the nucleotide sequences of this invention. The sequences are incorporated into constructions comprising the nucleotide sequences, up to and including the ATG (whilst leaving the second amino acid unmodified), or alternatively up to and including the GTC subsequent to the ATG (with the possibility of modifying the second amino acid of the transgene).

Expression of the nucleotide sequences in transgenic plants is driven by promoters shown to be functional in plants. The choice of promoter will vary depending on the temporal and spatial requirements for expression, and also depending on the target species. Thus, expression of the nucleotide sequences of this invention in leaves, in ears, in inflorescences (e.g. spikes, panicles, cobs, etc.), in roots, and/or seedlings is preferred. In many cases, however, protection against more than one type of insect pest is sought, and thus expression in multiple tissues is desirable. Although many promoters from dicotyledons have been shown to be operational in monocotyledons and vice versa, ideally dicotyledonous promoters are selected for expression in dicotyledons, and monocotyledonous promoters for expression in monocotyledons. However, there is no restriction to the provenance of selected promoters; it is sufficient that they are operational in driving the expression of the nucleotide sequences in the desired cell.

Preferred promoters that are expressed constitutively include promoters from genes encoding actin or ubiquitin and the CAMV 35S and 19S promoters. The nucleotide sequences of this invention can also be

expressed under the regulation of promoters that are chemically regulated. This enables the insecticidal toxins to be synthesized only when the crop plants are treated with the inducing chemicals.

A preferred category of promoters is that which is wound inducible. Numerous promoters have been described which are expressed at wound sites and also at the sites of phytopathogen infection. Ideally, such a promoter should only be active locally at the sites of infection, and in this way the insecticidal toxins only accumulate in cells which need to synthesize the insecticidal toxins to kill the invading insect pest. Preferred promoters of this kind include those described by Stanford et al. Mol. Gen. Genet. 215: 200-208 (1989), Xu et al. Plant Molec. Biol. 22: 573-588 (1993), Logemann et al. Plant Cell 1: 151-158 (1989), Rohrmeier & Lehle, Plant Molec. Biol. 22: 783-792 (1993), Firek et al. Plant Molec. Biol. 22: 129-142 (1993), and Warner et al. Plant J. 3: 191-201 (1993).

Especially preferred embodiments of the invention are transgenic plants expressing at least one of the nucleotide sequences of the invention in a root-preferred or root-specific fashion. Further preferred embodiments are transgenic plants expressing the nucleotide sequences in a wound-inducible or pathogen infection-inducible manner.

In addition to the selection of a suitable promoter, constructions for expression of an insecticidal toxin in plants require an appropriate transcription terminator to be attached downstream of the heterologous nucleotide sequence. Several such terminators are available and known in the art (e.g. tml from Agrobacterium, E9 from rbcs). Any available terminator known to function in plants can be used in the context of this invention.

Numerous other sequences can be incorporated into expression cassettes described in this invention. These include sequences which have been shown to enhance expression such as intron sequences (e.g. from Adh1 and

bronzel) and viral leader sequences (e.g. from TMV, MCMV and AMV).

It may be preferable to target expression of the nucleotide sequences of the present invention to different cellular localizations in the plant. In some cases, localization in the cytosol may be desirable, whereas in other cases, localization in some subcellular organelle may be preferred. Subcellular localization of transgene encoded enzymes is undertaken using techniques well known in the art Typically, the DNA encoding the target peptide from a known organelle-targeted gene product is manipulated and fused upstream of the nucleotide sequence. Many such target sequences are known for the chloroplast and their functioning in heterologous constructions has been shown. The expression of the nucleotide sequences of the present invention is also targeted to the endoplasmic reticulum or to the vacuoles of the host cells. Techniques to achieve this are well-known in the art.

Vectors suitable for plant transformation are described elsewhere in this specification. For Agrobacterium-mediated transformation, binary vectors or vectors carrying at least one T-DNA border sequence are suitable, whereas for direct gene transfer any vector is suitable and linear DNA containing only the construction of interest may be preferred. In the case of direct gene transfer, transformation with a single DNA species or co-transformation can be used (Schocher et al. Biotechnology 4: 1093-1096 (1986)). For both direct gene transfer and Agrobacterium-mediated transfer, transformation is usually (but not necessarily) undertaken with a selectable or screenable marker which may provide resistance to an antibiotic (kanamycin, hygromycin or methotrexate) or a herbicide (Basta). Examples of such markers are neomycin phosphotransferase, hygromycin phosphotransferase, dihydrofolate reductase, phosphinothricin acetyltransferase, 2, 2-dichloroproprionic acid dehalogenase, acetohydroxyacid

synthase, 5-enolpyruvyl-shikimate-phosphate synthase, haloarylnitrilase, protoporhyrinogen oxidase, acetyl-coenzyme A carboxylase, dihydropteroate synthase, chloramphenicol acetyl transferase, and •-glucuronidase. The choice of selectable or screenable marker for plant transformation is not, however, critical to the invention.

The recombinant DNA described above can be introduced into the plant cell in a number of art-recognized ways. Those skilled in the art will appreciate that the choice of method might depend on the type of plant targeted for transformation. Suitable methods of transforming plant cells include microinjection (Crossway et al., BioTechniques 4.,320-334 (1 986)), electroporation (Riggs et al., Proc. Natl. Acad, Sci. USA 83.,5602-5606 (1986), Agrobacterium-mediated transformation (Hinchee et al., Biotechnology 6:915-921 (1988); See also, lshida et al., Nature Biotechnology 14:745-750 (June 1996) (for maize transformation), direct gene transfer (Paszkowski et al., EMBO J. 3.2717-2722 (1984); Hayashimoto et al., Plant Physiol 93.857-863 (1990)(rice), and ballistic particle acceleration using devices available from Agracetus, Inc., Madison, Wisconsin and Dupont, Inc., Wilmington, Delaware (see, for example, Sanford et al., U.S. Patent 4,945,050; and McCabe et al., Biotechnology 6.923-926 (1988)). See also, Weissinger et al., Annual Rev Genet. 22.-421-477 (1988); Sanford et al., Particulate Science and Technology 5.27-37 (1987)(onion); Svab et al., Proc. Natl. Acad. Sci. USA 87.- 8526-8530 (1990) (tobacco chloroplast); Christou et al., Plant Physiol 87,671-674 (1988)(soybean); McCabe et al., BioTechnology 6.923-926 (1988)(soybean); Klein et al., Proc. Natl. Acad. Sci. USA, 85:4305-4309 (1988)(maize); Klein et al., BioTechnology 6.,559-563 (1988) (maize); Klein et al., Plant PhysioL 91.,440-444 (1988) (maize); Fromm et al., BioTechnology 8:833-839 (1 990); and Gordon-Kamm et al., Plant Cell 2: 603-618 (1990) (maize); Koziel et al.,

Biotechnology 1 1: 194-200 (1993) (maize); Shimamoto et al., Nature 338: 274-277 (1989) (rice); Christou et al., Biotechnology 9: 957-962 (1991) (rice); Datta et al., BioTechnology 8.736-740 (1990) (rice); European Patent Application EP 0 332 581 (orchardgrass and other Pooideae); Vasil et al., Biotechnology 1 1: 1553-1558 (1993) (wheat); Weeks et al., Plant Physiol. 102:1077-1084 (1993) (wheat); Wan et al., Plant Physiol. 104:37-48 (1994) (barley); Jahne et al., Theor. Appl. Genet. 89:525-533 (1994)(barley); Umbeck et al., BioTechnology 5:263-266 (1987) (cotton); Casas et al., Proc. Natl. Acad. Sci. USA 90:11212-11216 (Dec. 1993) (sorghum); Somers et al., BioTechnology 10:1 589-1594 (Dec. 1992) (oat); Torbert et al., Plant Cell Reports 14:635-640 (1995) (oat); Weeks et al., Plant Physiol. 102:1077-1084 (1993) (wheat); Chang et al., WO 94/13822 (wheat) and Nehra et al., The Plant Journal 5:285-297 (1994) (wheat). A particularly preferred set of embodiments for the introduction of recombinant DNA molecules into maize by microprojectile bombardment can be found in Koziel et al., Biotechnology 11: 194-200(1993), Hill et al., Euphytica 85:119-123 (1995) and Koziel et al., Annals of the New York Academy of Sciences 792:164-171 (1996). An additional preferred embodiment is the protoplast transformation method for maize as disclosed in EP 0 292 435. Transformation of plants can be undertaken with a single DNA species or multiple DNA species (i.e. co-transformation).

In another preferred embodiment, a nucleotide sequence of the present invention is directly transformed into the plastid genome. A major advantage of plastid transformation is that plastids are generally capable of expressing bacterial genes without substantial modification, and plastids are capable of expressing multiple open reading frames under control of a single promoter. Plastid transformation technology is extensively described in U.S. Patent Nos. 5,451,513, 5,545,817, and 5,545,818, in PCT application no. WO

95/16783, and in McBride et al. (1994) Proc. Natl. Acad. Sci. USA 91, 7301-7305. The basic technique for chloroplast transformation involves introducing regions of cloned plastid DNA flanking a selectable marker together with the gene of interest into a suitable target tissue, e.g., using biolistics or protoplast transformation (e.g., calcium chloride or PEG mediated transformation). The 1 to 1.5 kb flanking regions, termed targeting sequences, facilitate homologous recombination with the plastid genome and thus allow the replacement or modification of specific regions of the plastome. Initially, point mutations in the chloroplast 16S rRNA and rpsl2 genes conferring resistance to spectinomycin and/or streptomycin are utilized as selectable markers for transformation (Svab, Z., Hajdukiewicz, P., and Maliga, P. (1990) Proc. Nati. Acad. Sci. USA 87, 8526-8530; Staub, J. M., and Maliga, P. (1992) Plant Cell 4, 39-45). This resulted in stable homoplasmic transformants at a frequency of approximately one per 100 bombardments of target leaves. The presence of cloning sites between these markers allowed creation of a plastid targeting vector for introduction of foreign genes (Staub, J.M., and Maliga, P. (1993) EMBO J. 12, 601-606). Substantial increases in transformation frequency are obtained by replacement of the recessive RRNA or r-protein antibiotic resistance genes with a dominant selectable marker, the bacterial aada gene encoding the spectinomycin-detoxifying enzyme aminoglycoside-3' adenyltransferase (Svab, Z., and Maliga, P. (1993) Proc. Natl. Acad. Sci. USA 90, 913-917). Previously, this marker had been used successfully for high-frequency transformation of the plastid genome of the green alga Chlamydomonas reinhardtii (Goldschmidt-Clermont, M. (1991) Nucl. Acids Res. 19: 4083-4089). Other selectable markers useful for plastid transformation are known in the art and encompassed within the scope of the invention. Typically, approximately 15-20 cell division cycles following

transformation are required to reach a homoplastidic state. Plastid expression, in which genes are inserted by homologous recombination into all of the several thousand copies of the circular plastid genome present in each plant cell, takes advantage of the enormous copy number advantage over nuclear-expressed genes to permit expression levels that can readily exceed 10% of the total soluble plant protein. In a preferred embodiment, a nucleotide sequence of the present invention is inserted into a plastid targeting vector and transformed into the plastid genome of a desired plant host. Plants homoplastic for plastid genomes containing a nucleotide sequence of the present invention are obtained, and are preferentially capable of high expression of the nucleotide sequence.

Provisional Patent Application Ser. No. US 60/191806 filed March 24, 2000, is hereby incorporated by reference.